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Skin Substitutes

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On tissue engineering field the research about skin substitutes represents historically the most promising approach to heal acute and chronic skin wounds, reducing requirements for donor skin autografts. Aim of this chapter is to made the state of art of the most important skin substitutes and to resume the principal tissue engineering techniques for its in vitro reconstruction. In the first part we will pay the attention to the description of the evolutive course of skin substitutes, stressing the main steps happened thanks to the important inputs coming from biotechnologies progresses and clinical practice. Since then the research in bioengineering skin, moved by clinical pressing of reconstructive and burn surgery, made progresses trying to develop cutaneous substitutes very similar to native skin by a morpho-functional point of view. So it has been possible, starting from a 2X2 cm skin human biopsy, to realize completely autologous cutaneous substitutes not only composed of two many structures of skin, dermis and epidermis, but containing also other important components: microvascular network, micro nervous network, skin immonocompetent system and melanocyte system. The final goal is to develop effective and easy handling skin substitutes that could be reproduce human skin anatomy and physiology, introducing new advantages linked to successful grafting and then to satisfactory clinical results by functional and aesthetic point of view.

1. Introduction

Tissue Engineering (TE) is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". Tissue engineering has also been defined as "understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use."

Powerful recent developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies. Scientific advances in biomaterials, stem cells, growth and differentiation factors, and biomimetic environments have created unique opportunities to fabricate tissues in the laboratory from combinations of engineered extracellular matrices ("scaffolds"), cells, and biologically active

molecules. Among the major challenges now facing tissue engineering there is the need for more complex functionality with biomechanical stability in laboratory-grown tissues destined for transplantation.

Thus, the scientific and medical communities are working together to develop engineered tissues and regenerative approaches utilizing various combinations of stem and progenitor cells, biomaterials, growth factors, and gene therapy. However, after three decades, the potential to provide tissues and organs to millions of patients suffering from trauma, congenital defects, and chronic diseases has yet to be fully accomplished.

On the whole, tissue engineering appears to be the new frontier of medicine for its impact on regenerative and reconstructive procedures in humans. In fact, its ultimate goal is to develop powerful new therapies, namely "biological substitutes", for structural and functional disorders that have been proven to be difficult or impossible to tackle successfully with the current approaches of interventional medicine. In addition, providing "cell-to-tissue replacement parts" of the human body it can ultimately afford the irremediable shortage of transplantable organs.

Despite the rapid advancement of state of the art medically driven technologies, numerous challenges still exist for translating technology to the clinic including long and often undefined regulatory approval pathways, high translational costs, potential safety appropriate risk/benefit and cost/benefit ratios, and appropriate matching of technology and application. The latter may be one of the most difficult tasks faced by the biomedical engineer. Although development of general platform technologies can lead to major breakthroughs across multiple disciplines, conventional wisdom suggests rapid clinical translation and requires a systematic approach that involves first identifying and understanding a problem followed by engineering a focused solution. As technology rapidly advances, the bioengineer have more tools at their disposal than ever before, yet only if they are able to select the right tools and technologies, for society benefit.

Tissue engineered substitutes should have some essential characteristics which include: being easy to handle and apply to the injured site; provide vital barrier function with appropriate water flux; be readily adherent; have appropriate physical and mechanical properties; undergo controlled degradation; be sterile, non-toxic, non-antigenic; and evoke none or minimal inflammatory reactivity. Additionally, they should incorporate into the host with minimal scarring and pain and facilitate angiogenesis, while still being cost-effective.

The ability to create thick tissues is a major tissue engineering challenge, requiring the development of a suitable vascular supply. Current trends are seeing the utilization of cells seeded into hybrid matrix/scaffold systems to create in vitro vascular analogues. Approaches that aim to create vasculature in vitro include the use of biological extracellular matrices, co-culture of cells, incorporation of growth factors, culture in dynamic bioreactor environments, and combinations of these. Of particular interest are those approaches that aim to create bioengineered tissues in vitro that can be readily connected to the host's vasculature following implantation in order to maintain cell viability. A major consideration when developing a tissue engineering strategy for promoting repair and regeneration is to identify suitable sources of cells and mechanisms by which they can function and interact properly. These cells would also have to be abundant enough to be able to carry out the regeneration process completely.

Another obstacle to the large scale clinical application of cell therapy using conventional procedure is the extremely high cost of the treatment. The economic limit is due to the high cost of the actual manual operations for ex vivo cell expansion and differentiation in respect of the international regulations (Good laboratory and manufacture practices, GLP, GMP or Food and Drug Administration, FDA) that control the trading of clinical products.
Therefore there is a diffuse and pressing necessity to develop new methods and culture techniques that are able to control the cell micro-environment during both cell expansion and differentiation leading to repeatable clinical grade batches, operating in safety conditions and at low costs. This last point is often ignored but it is fundamental to make the clinical progresses available to everybody and not only to few people.

2. Anatomy of the skin

The skin is the largest organ of the body in vertebrates and is composed of the epidermis and dermis with a complex nerve and blood supply (Fig. 1). A third layer the hypodermis, is composed mainly of fat and a layer of loose connective tissue. These three layers play an important role in protecting the body from any mechanical damage such as wounding.

I. The epidermis is thin and highly cellular, composed mainly of keratinocytes and in the lower epidermal layers, melanocytes, for pigmentation. It does, however, have sufficient thickness to provide vital barrier function. Mammalian epidermis and its appendages (hair, nail, sweat and sebaceous glands) maintain homeostasis by constant recycling of the basal cell layer.

II. The dermis situated directly below the epidermis, constitutes the bulk of the skin and is composed of collagen with some elastin and glycosaminoglycans (GAG's). Hemidesmosomal structure are the main components of the basement membrane that mechanically stabilizes the interaction between epidermis and dermis. Basement membrane is an extremely complex and dynamic structure, which is responsible of the tight junction of the upper epidermis and underlying connective tissue. The dermis is a thicker tissue made essentially of a loose array of fibroblasts (mesenchymal cells) and vasculature (endothelial cells) forming an approximately 2-5 mm thick connective tissue sustaining the overlaid epidermis which is separated from the dermis by a 20 nm thick multilayered membrane: basement membrane. The dermal matrix, which provides considerable strength to skin by virtue of the arrangement of collagen fibers, has specialized components and structures. Collagenous mesh work is interwoven with varying contents of elastin fibers, proteoglycons (GAG being predominantly hyaluronic acid and dermatan sulfate with some chondroitin-6-sulphate and heparin sulphate), fibronectin and other components. (Metcalfe and Ferguson 2007)

III. The hypodermis, the final layer, contains adipose tissue that is well vascularised and contributes to both the thermoregulatory and mechanical properties of the skin.

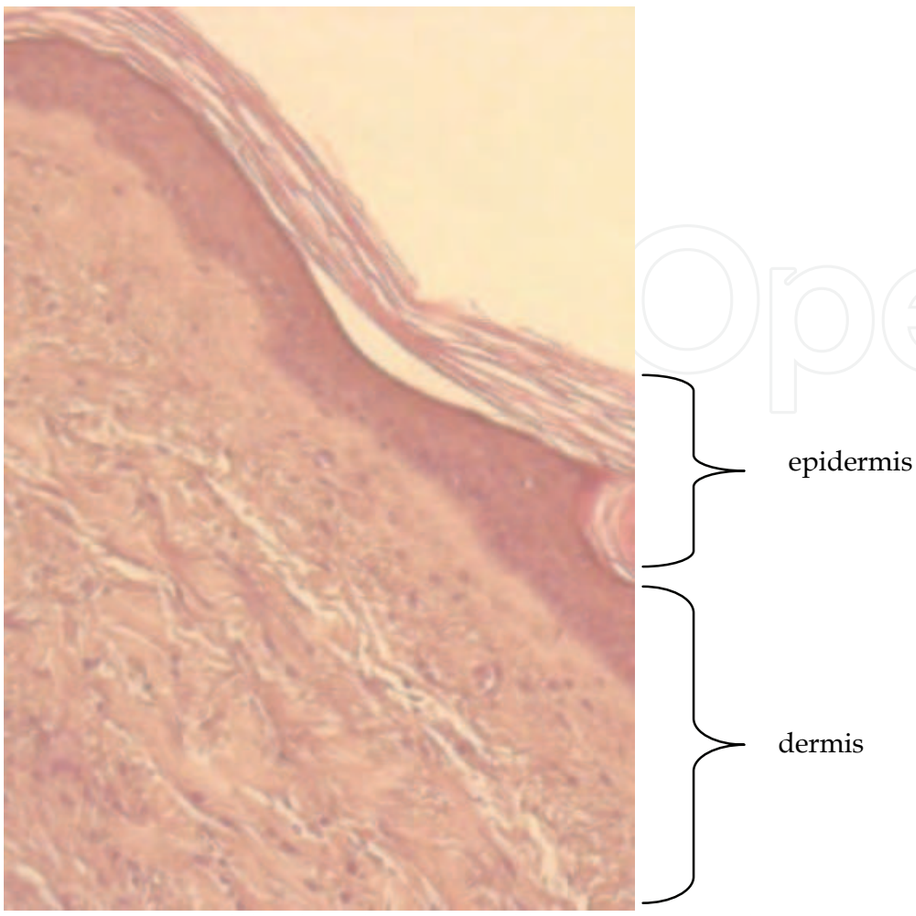


Fig. 1. Anatomy of the skin: dermis with fibroblasts and epidermis with keratinocytes

Skin is also composed of a series of appendages known as anexa, such as hair follicles, sweat glands and sebaceous glands generally embedded in the dermis. Because the skin serves as a protective barrier against the outside world, any break in it must be rapidly and efficiently amended. (Jennings RW and Hung TK 1982). The physiologic mechanism that coordinates the ordered repair of cutaneous tissues is generally referred to as wound healing and it consists in a well orchestrated series of temporary overlapping actions involving different cell and biochemical types. The process of wound healing involves interaction of a variety of different cell types and matrix components (R.A.F. Clark and P.M. Henson 1988). Different types of cells respond to environmental signals in a specific manner in order to carry out their genetic program of proliferation, differentiation and function. Cells synthesize different proteins necessary for their proliferation and migration, all of which are controlled in a phased manner (Zavan and Abatangelo 2004). Different phases are inflammation, granulation tissue formation, re-epithelialization, matrix production and remodelling Thus, each layer of skin plays its own pivotal role to play in the normal function of the skin, which otherwise can lead to several skin disorders. Healing of injured tissues involves both regeneration of the epidermis and repair of the dermis resulting in a scar tissue (M Calvin 1998).

3. Wound Healing

Wounds can arise either acutely from traumas such as burns, derive from surgical treatments such as skin graft harvesting or melanoma excision or also in a chronic fashion related to patho-physiological phenomena like insufficient vascularization, as in pressure wounds or diabetic foot ulcers. The underlying principles behind the use of skin substitutes to improve wound healing are valid for extensive wounds that cannot heal spontaneously because they are deep and affect a high percentage of the total body surface or for smaller wounds that cannot heal because of underlying factors (Auger and Lacroix 2009).
The normal acute wound healing process takes place in 4 steps (FIG.2):
1) hemostasis,
2) inflammation,
3) proliferation,
4) remodeling.

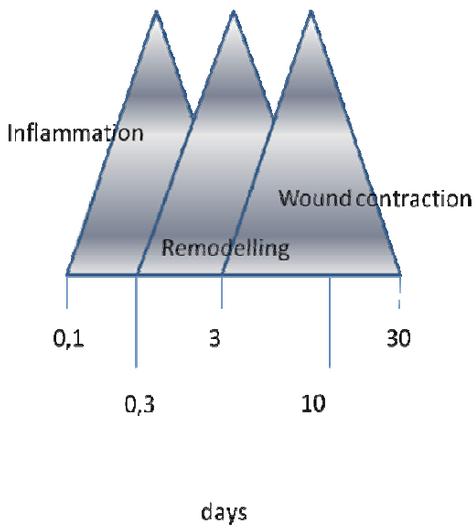


Fig. 2. Time table of wound healing process

Hemostasis
Unless there is severe arterial hemorrhage, hemostasis spontaneously begins immediately after the injury. It involves platelet aggregation, fibrin clot formation and activation of the coagulation pathways. Since the first aspects of wound treatment involve cleaning and debriding the wound, skin substitutes are rarely, if ever, used for hemostasis. However, it should be noted that chitosan dressings have been demonstrated to efficiently improve hemostasis compared to standard dressings, even with significant arterial hemorrhage [Garner and Brown R 2002]
Inflammation
Inflammation takes place 1–4 days after wounding. It involves the migration of neutrophils into the wound site, shortly followed by macrophages and later by lymphocytes. The fibrin clot matrix provides a three-dimensional initial scaffold through which immune cells will initially migrate to the wound and secrete a host of signaling molecules that will act as chemoattractants and growth factors. Platelets will produce factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-α (TGF-

α), transforming growth factor- β (TGF- β) and vascular endothelial growth factor (VEGF). [Werner and Grose 2003; Spiekstra et al. 2007].

Proliferation

Factors secreted during inflammation will trigger and drive the proliferation stage, which normally takes place between days 4 and 21 following wounding. During that stage, fibroblasts are stimulated by fibroblast growth factor (FGF) and PDGF to invade the wound site, and will produce xtracellular matrix (ECM) components, such as collagen, elastin and glycosaminoglycans, to generate the granulation tissue. Fibroblasts secrete also FGF that can, along with the VEGF secreted by platelets and neutrophils, act as an angiogenic factor to stimulate endothelial cell proliferation and migration and thus promote vascularization at the healing site. While fibroblasts and endothelial cells mostly invade the wound site from its bed, keratinocytes at the margin of the wound undergo a transient burst of proliferation that will sustain epithelialization over the wound (FIG.3)[Laplante AF et al. 2001]

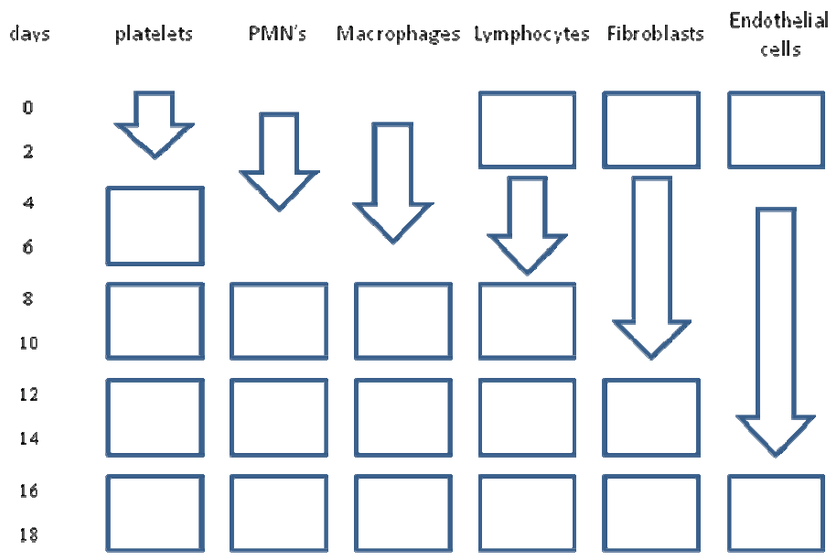


Fig. 3. Cells involved on wound healing

Remodeling

The last step of normal acute wound healing is remodeling, during which the ECM components will be modified by the balanced mechanisms of proteolysis and new matrix secretion, and during which the wound becomes gradually less vascularized. The initial granulation tissue is weak but will gain in strength over time because of remodeling effects such as the gradual replacement of immature type III collagen by mature type I collagen. The complete maturation of a wound may take months or even years to complete, depending on its initial extent and various factors that can affect the healing process [[Johnstone and Farley 2005]. Excessive contraction in large wounds can lead to contractures, while excess formation of scar tissue will form hypertrophic scars or even, if the scar tissue extends into the healthy tissue around the wound, a keloid scar. Wound contraction starts taking place around 6 days after injury and can last long after the epithelialization phase. The myofibroblast is considered to be the major cell responsible for contraction and is easily distinguished from normal fibroblasts by the presence of α-smooth

muscle actin (α-SMA). α-SMA protein expression is undetectable until day 6 after wounding, after subsequently increases gradually for 15 days in the healing process and fades after 4 weeks [Brunicardi et al 2005].

3.1 Clinical application

Today, surgical grafting of split-thickness autologous skin is the mainstay for treatment of fullthickness burns and is often used to treat chronic wounds of all kinds. The depth of tissue destruction and the need to replace different functions will determine the type of skin substitute used:

a) Rapid replacement of the epidermal layer is important to restore, as fast as possible, control over fluid loss, body temperature and protection against bacteria. Epidermal or bilayer substitutes that do not provide autologous keratinocytes or immunocompatible living tissue, such as cadaveric skin, xenografts and inert membranes like silicone, can only provide temporary coverage. They are indicated if availability and expected healing speed make them the best or the only option at the beginning of the treatment. However, live keratinocytes have to be present to achieve permanent wound coverage and restore the epidermal barrier. With epidermal substitutes, the presence of autologous living cells is thus necessary for proper healing of extensive areas where spontaneous epithelialization is impossible or would take too long to complete. Autologous living cells can also be desirable to improve the speed and/or quality of the healing in wounds that otherwise would be small enough to epithelialize on their own [Macri et Clarke 2009].

b) In cases where the dermal layer is still at least partially present even after debridement, epidermal substitutes that provide living autologous keratinocytes can be applied directly on the wound bed and eventually achieve permanent wound closure. However, in cases where the dermal layer has been destroyed, the application of even the best epidermal substitute is not enough to ensure optimal healing.The dermal layer is essential for better elasticity and mechanical resistance, the absence of epithelial-mesenchymal communication increases the frequency of developing fibrotic conditions [Sawicki et al 2005] and evidence indicates its presence allows highly complex ex vivo function of epidermal cells. Thus dermal reconstruction should be an essential part of the treatment in cases where none of the original dermis is left on the wound bed prepared for grafting. The presence of living fibroblasts is not a necessity in dermal substitutes. Such acellular products can be sufficient to provide a scaffold that will be repopulated, revascularized and remodeled with the patient's own fibroblasts and endothelial cells. However, the presence of fibroblasts in dermal substitutes helps wound healing by providing factors of the FGF family, by the secretion of the ECM responsible for its mechanical properties and by the structuring effect they have on the formation of capillaries during angiogenesis [Mitrani et al 2005]. Dermal substitutes provide a crucial component to help repair full-thickness damage, but they do not create a barrier effect and need to be covered with an epidermal substitute to that end.

Basically, there are three methods of skin repair and replacement:
First: autografting.

This is a widely used method of treating organ loss. For instance, it is an effective procedure for the short-term treatment of full thickness skin loss, especially meshed skin in severely burned patients. Although autologous skin grafts remain the first option in the current standard of care for burn patients, there are clinical circumstances where using the donor's own skin might not be appropriate for reasons related to the quantity needed, as in the case of massive tissue injury such as extensive burns or because of healing deficiencies that lead to chronic wounds. The clinicians then need to turn towards alternative materials that will offer appropriate properties for the type and extent of the wound to be treated [Machens et al. 2000]

Second: cadaver skin

It has offered an alternate pathway to skin replacement for many years. Peter Medawar, in his early pioneer work on transplantation immunobiology during the 1930s and 1940s, used skin transplants in immunoincompatible animal models and thus outlined very early in the history of transplantation medicine that rejection and progressive loss of tissue is a major drawback for cadaver skin replacements. In recent decades the consequences of a promiscuous multiculture have taught us that additional problems are inherited with this type of surgery: hepatitis and the Aids virus are risks which cannot be excluded and make cadaver skin transplantation more and more obsolete. The potential of cadaveric allograft skin to transmit disease has been recognized as an increasing problem since the 1980s. Alternatives to cadaveric skin are represented by native biological substitutes, with or without living cells resumed in table 1.[Auger and Lacroix 2009]

Product	Commercial name
Fresh cadaveric skin	
Cryopreserved cadaveric skin	AllodermTM
Glycerolized cadaveric skin	
Fresh xenografts Usually fresh porcine disinfected skin	
Cryopreserved amniotic membrane	AmniograftTM
Porcine small intestine submucosa	OasisTM, FortaFlexTM
Porcine skin	PermacolTM

Table 1. commercial natural skin

Third: in vitro reconstructed skin

This procedure relies on the hypothesis that tissue can, in principle, be cultured in vitro. During the several preceding decades, there have been breathtaking achievements in medical science in the field of in vitro cell cultivation, tissue engineering and new biopharmaceutical as well as recombinant genetic therapies. The skin has been the first tissue-engineered organ from the lab bench to the patient [Rheinwald and Green, 1975]. Rheinwald and Green took 3T3 cells derived from mouse embryos (the 3T3 cell line established by Rheinwald and Green in particular is known as 3T3-J2), irradiated them to eliminate their capacity to divide, and co-cultured them with epidermal keratinocytes,

sufficiently uppressing the differentiation of the latter to obtain cells with a high proliferative capacity (Hiroco, 2007). The greatest merit of cultured epidermis is that it enables the grafting of epidermal keratinocytes that have preserved sufficient proliferative capacity.

(Metcalfe) Engineering design specification in skin has relied upon the creation of both artificial dermal and epidermal components, which when combined produce a replacement of the skin, that can be grafted in place ([Supp and Boyce 2005]. Materials used as artificial ECM to date include those derived from naturally occurring materials and those manufactured synthetically [MacNeil 2007]. Examples of natural materials include polypeptides, GAG's, fibronectin, collagen, hydroxyapatites, hyaluronan, chitosan and alginates. These materials are advantageous in that they have low toxicity and a low chronic inflammatory response. Examples of synthetic materials include polyglycolide (PGA), polylactide (PLA) and polylactide-coglycolide (PLG), which have been used for sutures and meshes [[Metcalfe and Ferguson 2007]. Other examples include polytetrafluoroethylene (PTFE), polycaprolactone (PCL) and polyethylene terephthalate (PET). Matrices used routinely in therapeutic applications are made from polymers that are often resorbed by or degraded in the body. In the early days of tissue engineering, polymers were adapted from other surgical uses and over time have been shown to have deficiencies in terms of mechanical and degradation properties [Griffith 2002]. A greater challenge arises when functionalising these polymers into scaffolds that have defined shapes and a complex, porous internal architecture that can direct tissue growth [Griffith and Schwartz 2006].

Current skin constructs that are commercially available are generally based on the following techniques:

a) Epidermis: with only keratinocytes

The minimum requirement for a viable skin construct is to reestablish a barrier function to avoid infection and water loss. The horny layer of the epidermis (the product of terminal keratinocyte differentiation) plays this role. As mentioned above, after the breakthroughs of Rheinwald and Green in the mid '70s, the technology of cultured epithelial autograft (CEA) is now available to a large number of hospitals worldwide. In addition, cell culture parameters, harvest procedures and usage indications have been strictly defined and CEA production is also being performed on an industrial basis.

Epidermal substitutes

Commercial name	Cell source	biomaterial
EpicelTM	autologous keratinocytes from skin	petrolatum gauze backing
EpidexTM	autologous keratinocytes from hair follicles outer root sheath	silicone membrane
LaserskinTM,	autologous keratinocytes	esterified laser-perforated hyaluronic acid matrix
BioSeed-STM	Autologous oral mucosal cells	fibrin matrix
MyskinTM	autologous keratinocytes	specially treated silicone sheet

CellSprayTM	autologous keratinocytes	suspension for spray
CryoskinTM	Cryopreserved (viable cells) confluent allogeneic keratinocytes	gel-like proprietary chemical surface

The greatest merit of cultured epidermis is that it enables the grafting of epidermal keratinocytes that have preserved sufficient proliferative capacity. Whatever the circumstances, patients in whom epidermal damage has occurred all ultimately require an epidermis composed of epidermal keratinocytes. The only hope for patients with widespread epidermal damage, such as whole-body burns, is the localized capacity of surviving epidermal keratinocytes to divide and proliferate, and their treatment requires an extremely long period of time. Although autologous grafting of skin from other areas of the body is the surgical norm, the amount of skin that can be harvested is limited, and furthermore, new epidermal damage is created at the donor site. On the other hand, cultured epidermis is very thin, and its use is problematic in cases of deep skin damage such as for patients in whom the dermis has been completely destroyed. For this reason, other methods must be used in advance to create dermal tissue that can offer a favorable base for grafting. [Hichiro 2007]

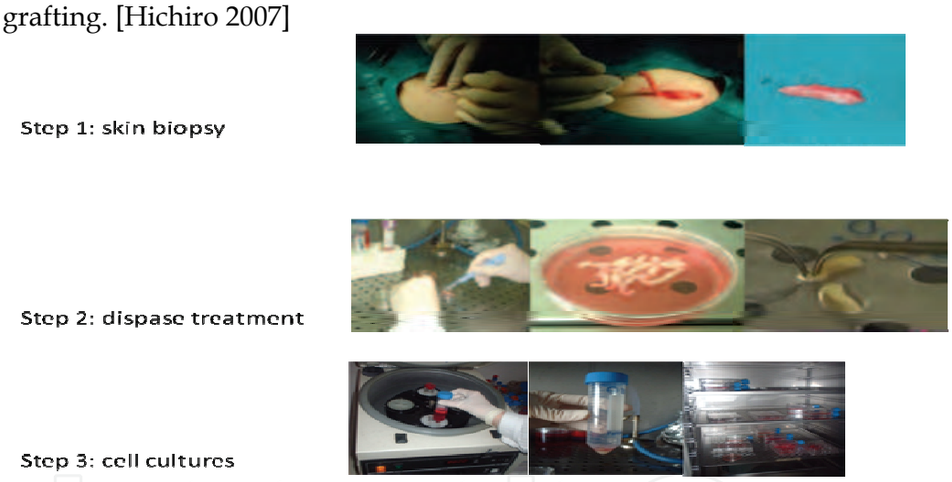
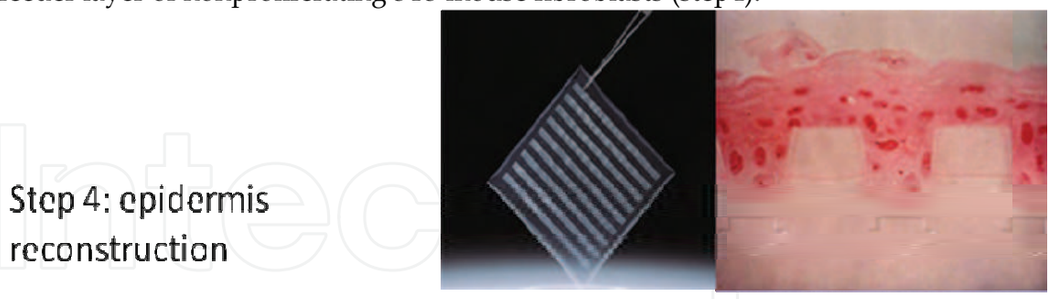


Fig. 4. Principal steps for in vitro epidermal skin reconstruction

Technique:
Keratinocytes are isolated from full-thickness skin biopsies (3×1 cm) (step1) obtained from healthy patients with ages ranging from 35 to 45 years. Keratinocytes are isolated and cultured according to a modified version of the Rheinwald and Green protocol. Briefly, dermal specimens are immersed in DMEM supplemented with 20% FBS and minced (step2).The small fragments obtained, are digested in 0.6 U/ml dispase II (Boehringer Mannheim) at 37° C for 1 h, at this time a manual separation between dermis and epidermis is performed (step2). The epidermis will be treated with trypsin ± EDTA (0.05%, 0.02 mM) solution (Seromed. Biochrom KG) for 10 min and the cells collected after a centrifugation and seeded in flask treated with a special keratinocytes attachment factors or in a feeder layer of 3T3 (step3).

For 3D coltures, keratinocytes are plated in secondary cultures at a density of 2 × 104 cells/cm² onto the biomaterials (e.g. hyaluronic acid: LaserSkin membrane) in the presence of a feeder layer of nonproliferating 3T3 mouse fibroblasts (step4).



b) Dermis: presences of fibroblasts.

Fibroblasts are mesenchymal cells that can be readily cultured in the laboratory and play a significant role in epithelial–mesenchymal interactions, secreting various growth factors and cytokines that have a direct effect on epidermal proliferation, differentiation and formation of extracellular matrix. They have been incorporated into various tissue-engineered products such as Dermagraft_ (Advanced BioHealing, La Jolla, CA, U.S.A.) and Apligraf_ (Novartis, Basel, Switzerland) and used for a variety of clinical applications, including the treatment of burns, chronic venous ulcers and several other clinical applications in dermatology and plastic surgery. Fibroblasts are a heterogeneous population of cells found in numerous tissues and are of mesenchymal origin. Fibroblasts from different anatomical sites all have similar morphology but DNA-microarray studies have demonstrated that fibroblasts in different anatomical sites have their own gene-expression profile and characteristic phenotypes, synthesizing extracellular matrix (ECM) proteins and cytokines in a site-specific manner. Dermal fibroblasts have numerous functions, not only in synthesizing and depositing ECM components, but also proliferation and migration in response to chemotactic, mitogenic and modulatory cytokines, and also autocrine and paracrine interactions [Wong et al 2007].

Fibroblasts used in tissue engineering may be allogenic or autologous. In contrast to allogenic cells, autologous fibroblasts carry no risks of rejection or risk of cross-infection.

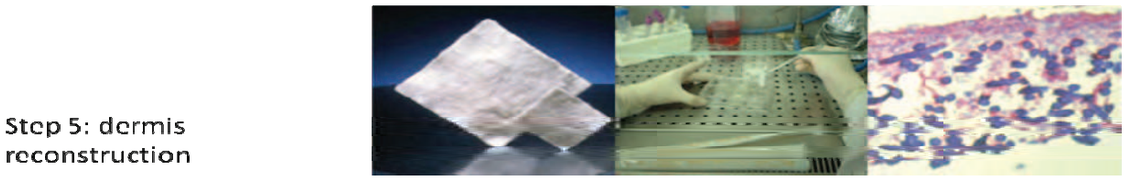
Cultured dermis using fibroblasts falls within this field, and has been produced with a number of materials. As the basic element, collagen or other biodegradable material has been used as a scaffold to create a tissue consisting of dermis-derived fibroblasts. Treatment using cultured dermis makes use of the wound-healing effects brought about by the physiologically active substances produced by fiobroblasts. Normally, the creation of granulation tissue mediated by localized inflammation is observed as a biological reaction at the site of skin loss. The reconstruction of connective tissue increases with time, mainly by the action of fibroblasts from the surrounding area, as does capillary regeneration. Cultured dermis, by enabling cultured fibroblasts to produce physiologically active substances that are important in the reconstruction of connective tissue, promotes favorable wound treatment. The intended effect of cultured dermis is the promotion of wound treatment by means of the physiologically active substances produced by fibroblasts contained within the culture. As results this treatment is characterized by the lack of any significant difference in outcome whether the fibroblasts used are autologous or allogeneic cells. In emergency use for burns, for example, allogeneic cultured dermis made in advance from allogeneic cells is

of high clinical utility. It will be necessary to solve the variety of concerns related to the use of living cells, as well as to demonstrate this method's clear therapeutic advantages over the use of pharmaceuticals alone through numerous examples of clinical application. [Hichiro 2007]

Dermal substitutes

Commercial name	Cell source	biomaterial
DermagraftTM	Cryopreserved (viable cells) allogeneic fibroblast	derived dermal matrix
Hyalograft 3DTM	autologous fibroblasts	Esterified hyaluronic acid matrix
AllodermTM		Acellular (freeze-dried) allogeneic dermis
OasisTM		porcine small intestine submucosa
EZ-DermTM		Aldehyde-crosslinked porcine dermal collagen
RepliformTM		Acellular human dermal allograft
CymetraTM		Micronized particulate acellular cadaveric dermal matrix
FortaFlexTM		porcine small intestine submucosa
BiobraneTM		Porcine collagen chemically bound to silicone/nylon membrane
MatridermTM		Acellular scaffold made with bovine collagen types I, III, V and elastin

Technique:
Human dermal fibroblasts are prepared according to a modified version of the Rheinwald & Green protocol. After epithelial sheet dispase removal, dermis was cut into small pieces (2–3 mm^2) and fibroblasts were isolated by sequential trypsin (0,05%) and collagenase type I digestion. Cells isolated are then cultured with DMEM medium supplemented with 10% Foetal Bovine Serum (FBS). At confluence fibroblasts are harvested and seeded at a density of 3 × 105/cm2 on squares of biomaterials (i.e. hyaluronan non woven meshes) (1.5 × 1.5 cm) in the above-mentioned medium containing sodium ascorbate (50 mg/mL) (step 5). The nonwoven squares could be fixed on culture plates either by means of stainless steel rings or through a fibrin clot.



c) Co-coltures of fibroblasts and keratinocytes (Bilayer substitutes).

After these first attempts, given that fibroblasts are the main constituent of the dermis and are normally used to grow keratinocytes, the concept of a co-culture was introduced in order to produce in vitro a composite skin replacement composed of an epithelial layer overlaid onto a dermal substitute. The presence of fibroblasts within the grafted region is thus a major advantage. Several lines of research are being pursued to define the best conditions of use. These include studies of allogenic fibroblast persistence in humans. Such fibroblasts persist in animals, but in humans, even if allogenic fibroblasts give similar results to autologous fibroblasts, their ultimate fate is unknown. Definition of fibroblast populations within the dermis has been undertaken. There is clear evidence that, depending on the anatomical region or the depth within the dermis, fibroblasts can be distinguished on the basis of functional criteria. It would be useful to have markers to select those cells that would be most efficient within dermal substitutes. At present despite active research in this field there is still no commercially available fibroblast marker. Lastly, the possibility of using mesenchymal stem cells collected from bone marrow or peripheral blood, which seem to participate in wound healing, is being evaluated. In addition to skin substitutes for permanent skin replacement, other applications can help to rapidly cover patients' wounds (despite eventual rejection of allogenic cells) or to accelerate healing. It is already known that allogenic epidermal cell cultures promote external ulcer healing, [Zavan and Abatangelo 2004] probably by secreting factors (matrix metalloproteinases, growth factors, etc.) that promote wound cleansing and stimulate the activity of cells present at the wound site. At the end, the importance of the scaffold for a dermal-like tissue must be stressed too. In fact, the design of a dermal-like tissue critically depends on the use of an ideal scaffold which must allow the dermis to develop into a three-dimensional architecture. One of the most important features of the scaffold is its porosity, which has to fit the neo-vessel ingrowth from the host tissue (angiogenesis) during the wound healing process. In addition, the biomaterial should be fully biocompatible and totally degradable being substituted by a full functional ECM in the long term. Finally, the material should be resistant to mechanical forces and easy to apply on the wound area.

The following guidelines emerged from the clinical experience:

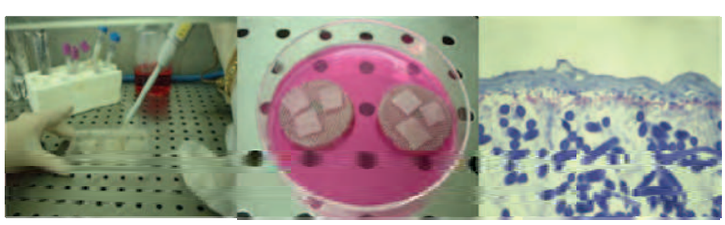
- The homogeneity of the cultured epidermal sheet is crucial for a good cosmetic result.
- One-step grafting of a cocultured dermis and epidermis is not yet satisfactory, and would be too lengthy for emergency use (e.g., burns).
- Preliminary grafting of a collagen matrix is hemostatic, reduces pain, and prepares the graft bed.
- Most importantly, the presence of living fibroblasts speeds up the reappearance of a functional neodermis (less than 1 year instead of 3 to 5 years when the graft bed is prepared only with cryopreserved cadaver skin).

Bilayer substitutes

Commercial name	Cell source	biomaterial
OrCelTM	Allogeneic keratinocytes seeded over dermal scaffold containing allogeneic fibroblasts	bovine collagen sponge
ApligrafTM	Allogeneic keratinocytes seeded over dermal scaffold containing allogeneic fibroblasts	bovine collagen sponge
TissueTechTM autograft system	Combination of Hyalograft 3DTM and LaserskinTM	
PermaDermTM	Autologous keratinocytes seeded onto dermal substitute made with autologous fibroblasts in bovine collagen matrix	bovine collagen matrix
IntegraTM		Temporary silicone epidermal substitute over dermal scaffold made of collagen and chondroitin-6 sulfate

The success of a skin equivalent graft–take requires the viability of fibroblasts contained within and the keratinocytes
adhering to the surface. When either keratinocytes or fibroblasts are directly introduced into a freshly made excisional
wound, there is rapid necrosis of these transferred cells. When components are put together in vitro and transferred
to an excisional wound site in vivo, the skin equivalent graft has optimal skinlike qualities.
Technique:
At confluence (10–12 days), pieces of membranes (1.5 × 1.5 cm) previously seed with keratinocytes (step 4) are laid down and fixed to the nonwoven meshes on which dermal fibroblasts had been grown for 15 days (step 5). The fibroblast–keratinocyte composite cultures were laid down carefully on sterile stainless steel grids and then cultivated for 15 days at the air–liquid interface (step 6).

Step 6: bilayer reconstruction



4. Conclusion

The many studies conducted so far reveal that Tissue Engineering of the skin is only at the beginning of its use in human applications. Burns patients were the first targets for such tissue substitutes, then chronic diseases, such as venous ulcers, have followed. The more experience is gained from the surgeon, the more feedback for the basic scientist to improve the product and to broaden clinical indications. Nowadays, progress in cell culture and biomedical material technologies have added two important spare parts: epidermis and dermis to the surgeon's toolbox which can be reconstituted in the laboratory from small biopsies of the same recipient. Other parts will follow in a few years, with the final aim to generate a full transplantable replica of the skin with adnexa and vasculature.

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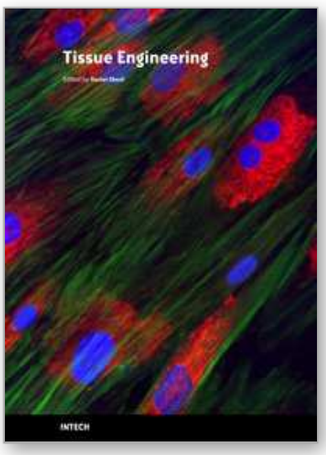

Tissue Engineering

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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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